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MEMBRANE INSERTION OF PROTHROMBIN

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SUMMARY: The nature of the interaction of prothrombin with liposomes was examined by labelling with the apolar reagent 5-[^{125}I] iodonaphthyl-1-azide (INA). When liposomes containing phosphatidylcholine were incubated with prothrombin and INA, the amount of incorporated label was greater in the absence of added calcium. Increasing the phosphatidylserine content of the liposomes, irrespective of Ca^{++}, resulted in a significant increase in the labelling of prothrombin by INA. Since INA is confined to the lipid core of the bilayer, these results strongly suggest that prothrombin binds to liposomes, and penetrates into the lipid bilayer.

In the blood coagulation cascade, several steps require the formation of membrane-bound complexes. It is known that thrombin formation is considerably enhanced by the interaction of the zymogen with negatively charged phospholipids (1). However, the molecular details of this reaction are not clearly understood and are the subject of controversy.

It is commonly claimed that Ca^{++} is essential for binding, and that the region of Fragment 1 of prothrombin is bound to the negative phosphatidylserine (PS), mainly by salt bridges, with the long axis of the prolate ellipsoid molecule lying normal to the plane of the lipid bilayer. It is not clear whether penetration into the lipid bilayer is required for the enhanced susceptibility of prothrombin toward activation. Lecompte et al. (2,3,4) obtained evidence that prothrombin and some of its fragments can penetrate into phospholipid monolayers. Furthermore, these authors found the interaction with PS-containing monolayers to occur even in the absence of calcium. Mayer et al. (5), in similar experiments, could not detect penetration. On the other hand, Cuypers

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et al. (6) studied the association of prothrombin with multibilayers by ellipsometry. They concluded that the interaction must be more complex than that of simple salt bridge formation.

In the present study, we have examined the possibility that direct penetration of prothrombin into the lipid bilayer could be detected by means of photolabelling with the apolar azide 5-[^{125}I]-iodonaphthalene-1-azide (INA). Previous studies have shown that INA has a high partitition coefficient into the liquid lipid phase of liposomes and membranes (7). Upon photolysis, the generated nitrene is confined to the lipid phase of the membrane where it binds covalently to lipid-embedded domains of proteins and to fatty acyl chains of the phospholipids. Our results indicated that prothrombin, in the presence of liposomes, was labelled by INA and that the labelling was enhanced as a function of PS content both in the presence and in the absence of Ca^{++}.

MATERIALS AND METHODS

Prothrombin was purified according to Dode et al. (8) and its purity checked by electrophoresis. Prothrombin concentration was determined by ultraviolet absorbance using an $\mathcal{E}^{1\%}_{280\ nm} = 14$.

The phospholipid PS, phosphatidylcholine (PC), and mixtures of both were dispersed in 1mM Tris, 0.15M NaCl pH 7.8 and sonicated for 1 hour in a bath-type sonicator (Laboratory supplies Co., Inc. 300 Watts) to yield single lamellar liposomes. The vesicles obtained (1mg/ml) were then incubated at room temperature for 1 hour, with prothrombin at a final concentration of 70 μg/ml in the presence and absence of 2.0 mM Ca^{++}. Under subdued light, 2 μl of (3x10 cpm) of an ethanol solution of ^{125}I-INA as prepared according to Raviv et al. (9) was added to 50 μl of the prothrombin liposome suspension. The reaction mixture was incubated for 4 min at 20°C and then irradiated for 5 min as previously described (7).

Electrophoresis in 5-15% gradient polyacrylamide gels in the presence of SDS (SDS-PAGE) was carried out according to Laemmli (10) with minor modifications. Sample buffer x4 consisted of 40% sucrose, 15% β-mercaptoethanol, 9% w/v SDS and 0.19 M Tris pH 6.8. To this, solid urea was added such that upon addition of the sample, the final urea concentration was 4 M. To eliminate proteolysis, samples were prepared by first bringing the sample buffer to a boil, then adding the sample with rapid mixing, and boiling for 2 min.

Autoradiography of the dried gels was performed using Curix RP-2 X-ray film (Agfa-Gevaert). Exposure was for 10 hours.

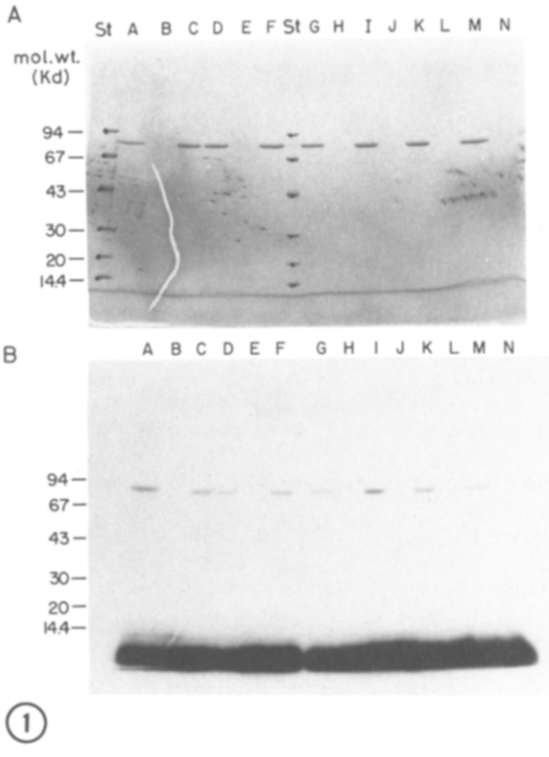
RESULTS AND DISCUSSION

Prothrombin was incubated with liposomes of different composition for one hour at room temperature. Conditions were such that the protein was saturated

with respect to the lipid. Subsequently, INA was added to a final concentration of 10 μM. After an additional 4 min. incubation, the solution was irradiated for 5 min. and then analyzed by SDS-PAGE. Coomassie blue R staining of the gel (Fig. 1A), revealed the presence of a single protein of mol.wt. 72000 daltons. These results indicated that the zymogen was stable to the photolysis conditions.

The autoradiogram of the Coomassie stained gel (Fig. 1A) is shown in Fig. 1B. Prothrombin (72 kd) and the phospholipids running at the front, were labelled by ^{125}I-INA. The actual counts associated with the protein were measured by cutting out the Coomassie stained protein band and determining the [^{125}I] content in a gamma counter. The results are shown in Fig. 2. In the presence of PC liposomes, incorporation of INA into prothrombin was observed to be higher in the absence of added Ca^{++}. Increasing the mole % of PS in the liposomes significantly increased the isotope incorporation irrespective of the presence of added calcium. When liposomes contained only PS, essentially the same high degree of labelling was observed either with or without added Ca^{++}.

Systematic studies on the effect of the INA concentration on the selectivity of membrane protein labelling indicated that at INA concentrations used in the experiments reported herein, the photogenerated nitrene was confined to the lipid core of the phospholipid bilayer (9). Therefore, the present findings strongly suggest that prothrombin is interacting with the liposomes such that a segment or segments of the protein come in contact with the apolar regions of the lipid bilayer where they become accessible to the apolar nitrene. It seems unlikely that soluble prothrombin is labelled directly by INA. If this were the case, we would not have expected that the degree of label incorporation would depend on the liposome composition (Fig. 2). The addition of INA directly to prothrombin, in the absence of liposomes, does not represent a valid control. This because, in the absence of a lipid phase, INA precipitates and could become nonspecifically adsorbed onto the protein leading to a labelling artifact. Only two soluble proteins have been found to be labelled by INA when photolysis is performed with intact cells or in the presence of liposomes. They are serum



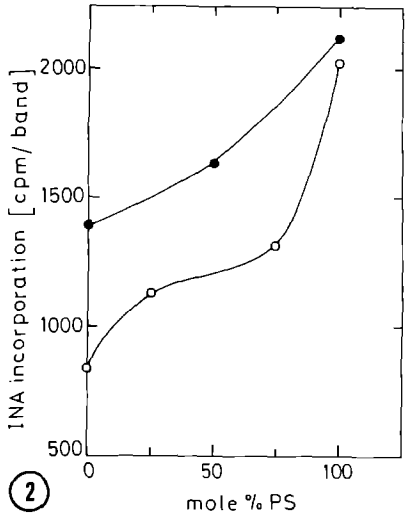


Figure 1. The staining and labelling patterns of prothrombin-liposome mixtures, following photolysis in the presence of [^{125}I]-INA. (A) Coomassie blue R staining pattern. Lanes A,C,D,F and G represent mixtures, in 2 mM Ca^{++}, of prothrombin and liposomes containing 100, 75, 60, 25 and 0 mole % PS in PC, respectively. Lanes I,K and M represent mixtures without Ca^{++} of prothrombin and liposomes containing 100, 50, 0 mole % PS in PC, respectively. Liposomes without prothrombin containing 100, 50, 0 mole % PS in PC are represented respectively in lanes B,E and H in the presence of Ca^{++} and J,L and N in the absence of Ca^{++}. Lanes marked St contain a set of standards with molecular weights indicated on the left side of the figure. (B) Autoradiogram of the prothrombin-vesicle mixtures following photolysis in the presence of [^{125}I]-INA. The content of each lane is identical to figure 1 A.

Figure 2. [^{125}I]-INA incorporated into prothrombin in the presence (o) or absence of Ca^{++} (•), as a function of the PS content of the liposomes.

albumin and apohemoglobin. Both contain binding sites for large apolar molecules, which could accomodate a molecule such as INA. Therefore our data indicate that the INA labelling of prothrombin in the presence of neutral PC liposomes is either due to the presence of a site designed to bind large apolar molecules or that prothrombin binds by such a site to the liposome surface. The decreased labelling observed on the addition of Ca^{++} may be due to the fact that

this ion alters the protein conformation as has been reported by others (11,12), thereby decreasing either the accessibility of the INA to the apolar binding site or reducing the interaction of such an apolar segment with the liposomes. It is unlikely that calcium affects the neutral liposome directly.

The enhancement of the labelling of prothrombin with liposomes containing increasing levels of PS in the absence of added Ca^{++}, suggested that the protein might interact by cationic amino acid side chains in the vicinity of the apolar site as has been described in prethrombin (13). The formation of ion-pairs with the anionic PS, would then increase the accessibility of the protein to labelling from within the lipid core.

The decreased incorporation of INA into prothrombin observed in PC-PS liposomes in the presence of added Ca^{++}, is probably due to: a) the effect of calcium on the prothrombin per se as described above; b) the reduction in the effective charge of the liposome due to the formation of PS-Ca^{++} ion-pairs; c) the formation of ternary ion-pairs between PS-Ca^{++}-prothrombin carboxylate groups. However, even in the presence of calcium, an increase in the mole % PS resulted in enhanced label incorporation. This indicated that as previously reported in monolayers (2,4), the negative PS enhanced penetration of the protein into the lipid bilayer.

It may be significant, furthermore, that the degree of INA incorporation into prothrombin in the presence of Ca^{++} showed a bimodal behaviour with respect to the mole % of PS. Low levels of PS (between 0 and 25%) increased incorporation, then a plateau was observed between 25 and 75%, followed by a rapid rise at PS levels above 75%. This is suggestive of several modes of binding which depend on surface charge density.

Under physiological conditions, the plasma calcium concentration is on the order of 1-2 mM. PS has generally been reported to be present in the internal monolayer of the plasma membrane bilayer. Therefore, conditions would be closer to those of the PC liposomes in the presence of Ca^{++}, (Fig. 2). Platelet activation or conditions that lead to the exposure of prothrombin to a membrane with a higher PS content, would lead to enhanced penetration and binding by ternary

ion pairs. This could make the zymogen more accessible to the proteolysis reaction at the cell surface. Prothrombin has been reported (13) to consist of 3 discrete domains: Fragment 1, which is highly negative, in part due to the γ-carboxyglutamic residues; Fragment 2, which is slightly negative, bridges prethrombin and Fragment 1; while prethrombin is positive and contains a high content of hydrophobic apolar amino acids. From the present results, it is likely that the prethrombin domain is that which penetrates into the lipid bilayer (2,4). The finding that prothrombin can be labelled by INA will permit the future determination of the actual domains of the protein that are labelled under different conditions. This should clarify many of the details currently unknown.

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